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METHOD FOR GENE TRANSFER OF PLANTS
[Verfahren zum Gentransfer von Pflanzen]

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This invention relates to a method of gene transfer in plants /2* by means of isolated, immature, and in vitro cultured pollen grains seeds produced from them, and propagation products of same.

There are already various techniques for gene transfer into plants. Each has its advantages and disadvantages (Goodman et al., Science 236: 48-53, 1987). At present, *Agrobacterium tumefaciens* is the most common vector. However, it is limited to a certain host range. The use of *A. tumefaciens* as a vector is still dependent on the regeneration in vitro of cultured somatic cells, in order to produce transgenic plants that are capable of forming transgenic progeny. Direct gene transfer by electroporation, liposomes, microinjection, and other physicochemical methods is highly dependent on the use of protoplasts as target cells. Regeneration from protoplasts is difficult, however, and even impossible in many species.

Another target cell, the pollen grain, has been suggested as an alternative to these gene transfer methods. According to Hess D., Plenum Press, New York, 519 to 537, 1975, the mature pollen is capable of taking up foreign DNA and bring this foreign DNA into the egg cell as a "supervector," using natural pollination and fertilization. Similarly, X-rayed pollen is said to be capable of transporting fragments of the irradiated genome into the egg cell (Pandey, Nature 256: 310-313, 1975). DeWet (WO 85/01856) reports that

* Numbers in the margin indicate pagination in the foreign text.

corn pollen that is treated with exogenous DNA takes up this DNA upon germination and transports it into the egg cell after fertilization.

Despite the potentially great importance of the gene transfer into and by pollen, there has not yet been a case in which the results of Hess, Pandey, and DeWet have been reproduced in other laboratories. Thus, for example, Engvild (Theor. Appl. Genet 69: 457-461, 1985) were unable to reproduce the experiments of Pandey. In the experiments of Hess [1975] and of DeWet, the uptake of exogenous DNA into the pollen grain and the genetic and molecular proof of transferred DNA is the critical point in their reasoning. Phenotypical and physiological proofs are insufficient (Hess in: Genetic Manipulation in Plant Breeding, de Gruyter, Berlin, New York 803-811, 1986). Experiments by Sanford, et al. (Biotechnology and Exology of Pollen (Mulcaby D., ed.), Springer, Heidelberg, New York, 71-75, 1968) also showed that co-culturing of mature *Nicotiana langsdorfii* pollen with agrobacteria failed to result in gene transfer into pollen. In extensive experimentation, Negrutiu, Heberle-Bors, and Potrykus (loc. cit. 65 to 70, 1986) were unable to transfer the neomycinphosphotransferase gene, which imparts resistance to kanamycin into mature pollen (Shillito et al., Biotechnology 3:1,099-1,103, 1985). Thus, using the latest methods of the prior art, it was not possible to confirm the statements of Hess and DeWet. The inability to reproduce the results of Hess and DeWet can be explained by the fact that as soon as they are placed in an

aqueous medium, which is required for the gene transfer, mature pollen grains begin to form a pollen tube. Obviously, they are no longer fertile at this time or the time available for the gene transfer is too short.

In Planta 170: 141-143 [1987], Pareddy, et al. describe the cultivation and maturation of immature corn tassels (male inflorescence) in vitro. The isolate mature pollen was used for pollination and seeds were taken from the pollinated plants. No proof of successful fertilization by means of genetic markers could be obtained.

Surprisingly, it was found that immature pollen grains can be cultured without the natural nutrient tissue containing them, that during various stages of maturation foreign genes can be introduced into these isolated immature pollen grains, and that plants can be pollinated and fertilized with the mature pollen grains to bring about normal seed formation, germination, and propagation.

Thus, the subject matter of the present invention is a method of gene transfer into plants, which is characterized in that

a) immature pollen grains are isolated from stamens in nutrient solution and the surrounding tissue is removed,

b) the isolated immature pollen grains are cultured in a /3 nutrient solution,

c) foreign genetic material is transferred into the pollen grains during the in vitro culturing and maturation,

d) the transformed pollen grains are brought to complete maturation in vitro,

e) receiver plants are pollinated with the transformed pollen grains and seeds are obtained from said plants.

The gene transfer into isolated, immature pollen grains, the in vitro maturation, and the use of pollen as a universal supervector open up a completely new strategy for gene transfer into plants. Compared to other methods, it is much simpler, since the cell culture phase is greatly shortened and regeneration, with the unpleasant accompanying phenomenon of somaclonal variation, is eliminated. Using this novel method, it is possible to transform even those plants in which successful gene transfer has been impossible in the past: Thus, for example, many species of grains, legumes, and trees cannot be regenerated from single cells or protoplasts.

The potential benefits from gene transfer technology are of great economic, ecological, and social value. An effort is being made to alter plants genetically in such a way as to increase yields, to make plants more resistant to diseases and pests, to make them tolerant to cold, heat, drought, salinization, and lack of nutrients, to give them greater nutrient qualities, to have them produce new raw materials for industry, and to make them fix their own nitrogen or become independent of fertilizers in some other way.

Essential to the invention is that pollen grains are cultured without surrounding tissue, i.e., they are isolated, so that the

genetic material that is to be transferred, coupled to a vector or naked, is in direct contact with the pollen grain. If the complete male inflorescence is cultured, it is not possible, due to the obstruction by the extensive surrounding tissue, using conventional transfer methods (*A. tumefaciens*, electroporation, microinjection, etc.) to transfer genes into the pollen grains.

An additional essential feature of this invention is the use of immature pollen grains. In this way, the entire time of the in vitro maturation is available for the gene transfer. The transfer into the immature pollen grain can occur in various stages of maturity, depending on the species of plant. In particular in gene transfer with vectors, it is important that the genetic material pass through as few cell walls as possible, in order to enter the genome of the sperm nucleus and become part of the zygote genome upon fertilization. The transfer preferably occurs when the microspores are in their uninucleate stage, but it can also occur during the first pollen mitosis, in the early binucleate stage, as long as the generative cell is still attached to the pollen wall, or additionally, in plants whose pollen is trinucleate in the mature stage (grains), in the stage just before or during the second pollen mitosis.

In particular, the method is carried out as follows, with tobacco (*Nicotiana tabacum*) being used as a model system. The system is applicable to all plants that can be propagated by pollination, in

particular to monocotyledonous and dicotyledonous crop plants such as wheat, corn, rice, legumes, oilseeds, vegetable plants, fruit plants, and forest plants.

In a preliminary experiment, the development stage of the pollen of the desired plant is determined in the usual manner by isolation of an anther, preparation of a squash preparation, and observation under the microscope after the addition of carmine acetic acid, for example.

Once the pollen has reached the desired stage, the pollen grains are isolated under aseptic conditions, by squeezing them out of the anthers in a nutrient medium, passing them through a sieve, washing them, centrifuging the, and resuspending them.

For pollen grains in their earlier development stage, the cell density should be higher than for pollen grains in a later stage of development. In the case of tobacco, cell density of binucleate pollen grains is approximately 10^5/ml, for uninucleate microspores it is approximately twice as high.

The nutrient medium that is used contains all the essential nutrient and growth substances for culturing and maintaining the maturation capability of the pollen grains. Depending on the plant, various compositions may result, so that it fulfils the function of the nourishing tissue (tapetum). The main constituents contained in the nutrient medium are sugar, nutrient substances, mineral salts, and vitamins, the pH being approximately 6.5 to 7.5. /4

In the case of uninucleate microspores, culturing is carried out up to the binucleate stage in a medium that is considerably enriched with sugar, e.g., sucrose, and additional nutrient substances. For example, the additional nutrients may be added in the form of coconut water. If the pollen grains have reached the binucleate stage, they can be transferred to a less nutrient-rich medium.

After the in vitro maturation has ended, the pollen grains are gathered for pollination. For this purpose, they are centrifuged off and washed and for pollination they are placed on flowers, from which the anthers have been removed, in an aqueous medium or dried. In a conventional manner, seeds that are capable of germination are obtained from the plants that have been germinated with the in vitro matured pollen grains.

For gene transfer during the in vitro culturing, the foreign genetic material to be transferred can be introduced into the pollen grains in conjunction with a customary vector, such as *A. tumefaciens,* or as naked DNA by direct transfer by means of electroporation, microinjection, or some other physicochemical method. The term "foreign genetic material" means any genetic material originating from outside the pollen grain that is to be transformed. After successful maturation, the pollen grains are harvested for pollination in the manner described above.

As proof of successful in vitro maturation, pollen grains from a tobacco plant were matured with two marker genes in vitro and normal wild type plants were pollinated with these pollen grains. The seeds that were obtained were sterilized and brought to germination in a germination medium selective, for example, to kanamycin resistance. The Mendelian segregation of the marker genes was demonstrated by counting the seedlings in the selective medium. This is proof that it was the in vitro matured pollen grains that were responsible for the fertilization and some pollen contamination from other plants.

As proof of the expression of the foreign gene introduced by transformation during the in vitro culturing, the chloramphenicol acetyltransferase activity (CAT activity) was detected in an enzyme assay in a homogenate of the transformed pollen grains.

Example 1: Determination of the pollen development stage.

Tobacco flowers were harvested in various lengths, the anthers isolated aseptically, one of the five anthers was placed on a slide, along with a drop of carmine acetic acid (4% carmine in 45% acetic acid) and a squash preparation was produced. The development stage of the pollen grains was determined under the microscope after 1/2 h.

Example 2: Isolation of the pollen grains

The tobacco pollen grains were isolated by squeezing the anthers carefully under aseptic conditions with a glass rod in AMGLU medium (1) and a microscopy mortar and the resulting pollen suspension was passed through a 75 µm sieve and washed twice in AMGLU medium.

Finally, the pollen suspension was centrifuged at 6,500 rpm in an Eppendorf centrifuge.

Example 3: Pollen culture for maturation of early binucleate pollen grains

Early binucleate tobacco pollen grains were isolated and the cell density adjusted to 10^5/ml in AMGLU medium. 1 ml of the pollen suspension was cultured in the dark in 35-mm petri dishes at 25°C. After 3 to 5 days, depending on the exact development stage of the pollen grains, the pollen grains were mature and could be harvested.

Example 4: Pollen culture for maturation of uninucleate microspores

Uninucleate tobacco spores were cultured in MR24 medium (2) at a cell density of $2x10^5$/ml. As soon as the pollen grains had reached the binucleate stage (after 2 to 5 days, depending on the exact age), they were centrifuged and further cultured in MIS medium (3) until maturation was complete.

Example 5: Pollination and proof of fertilization

The tobacco pollen grains were centrifuged off, washed in BK medium (Brewbaker and Kwack 1963), and the cell density was adjusted to $1.25X10^5$/ml. From flowers that were just opening (red flower tip), /5 the still closed anthers were removed. As soon as the flower had opened, a 4 µl drop of the pollen suspension was placed, using a 20 µl pipette, on the stigma of the flower, in such a way that the stigma was completely covered with pollen suspension. These operations were carried out without air movement and with other

plants of the same species at a distance. As soon as the drop had dried on the stigma, the stigma and stylus were covered with a 4 cm long piece of straw to prevent cross-fertilization. The plants were then returned to the greenhouse.

As proof that the in vitro matured pollen grains had carried out the pollination and fertilization, pollen grains of a transgenic plant were matured in vitro and normal wild type plants were pollinated with these pollen grains. As a marker gene, the transgenic plant contained the neomycin phosphotransferase gene (resistance to kanamycin) and the nopalinsynthase gene. Reciprocal crossing was also carried out.

Example 6: Seed harvest, seed germination, and genetic assay

The mature seed capsules obtained from Example 5 (brown and dry) were harvested and the seeds isolated by cutting off the tip of the capsule and pouring the seed grains directly into an Eppendorf tube. The seeds were surface sterilized in a NaOCl solution (3% free chlorine) for 5 min, washed twice with sterile water, and placed on a seed germination medium with kanamycin (4). After 4 weeks, the number of Kan^R and Kan^S seedlings was counted. In the crossing experiment above, the two reciprocal crossings produced a segregation of Kan^R:Kan^S = 1:1. Seedlings that grew without kanamycin had a segregation of Nos^+:Nos^- = 1:1 in a high-voltage paper electrophoresis test for nopalin.

Example 7: Transient expression of CAT gene

Agrobacteria (*A. tumefaciens* without tumor genes, with CAT gene coupled to a 35S promoter) were pre-incubated in Luria broth (6) for 1 day. Pollen grains in the early binucleate stage were isolated and cultivated in AMGLU medium. The bacterial suspension was adjusted with AMGLU medium to an OD_{580} of 0.2. After additional dilution with AMGLU medium of 1:10, 20µl of the bacterial suspension was added to 1 ml of the pollen suspension. After 24 h co-culturing, 1 µl claforan (1 g/2ml) per ml was added to kill the agrobacteria. After two additional days, the pollen grains were harvested and prepared in extract. For this purpose, 1.5 ml calcium washing solution (5), pH 5.6, was added per ml pollen suspension, centrifuged as 4,000 rpm for 5 min, and washed with a Tris buffer (0.25 M, pH 7.8). After centrifugation, the pollen pellet was mixed with 200 µl Tris buffer and homogenized by ultrasound (3 x 15 s) on ice. After 10 min on ice, the material was centrifuged and the supernatant kept at -20°C.

The CAT assay was then carried out as described by Sleigh (Anal. Biochem., 56: 251-256, 1986). 30 µl extract was mixed with 20 µl chloramphenicol (8 mM), 30 µl Tris buffer, and 20 µl ^{14}C-tagged acetyl CoA (5 µCi/ml in 0.5 mM cold acetyl CoA). After incubation at 37°C for 1 h, the acetylated chloramphenicol was extracted by shaking with ethyl acetate (2 x 100 µl) and the radioactivity measured in a scintillation counter.

Radioactivity (cpm) in the CAT assay of extracts from tobacco pollen after co-culturing with *A. tumefaciens*

	cpm
pollen in AMGLU medium	400
pollen with Agrobacteria in AMGLU medium	6000
pollen with acetosyringon-activated agrobacteria in AMGLU medium	6000
only agrobacteria in AMGLU medium	500
only acetosyringon-activated agrobacteria in AMGLU medium	500

The strong radioactivity signal shows that the agrobacteria have infected the pollen grains and the T-DNA must have entered the nucleus of the growing cell for expression (transcription).

As an additional control, to show that the agrobacteria /6 themselves had no CAT activity, an agrobacteria culture in Luria broth had no CAT activity over a complete growth cycle.

Culture media:

(1) AMGLU medium
- Miller's macrosalts (7)
- MS microsalts (8)
- Sucrose (0.25 M)
- Glutamine (440 mg/liter)
- pH 7

(2) MR24-medium
- MS macrosalts
- MS microsalts
- Sucrose (0.5 M)
- Glutamine (440 mg/liter)
- Coconut water (2 vol.-%)
- Lactalbumin hydrolysate (200 mg/liter)
- Inositol (100 mg/liter)
- pH 7

(3) M1S-medium

Miller's macrosalts
MS microsalts
FeEDTA (10^{-4} M)
Sucrose (0.25 M)
pH 7 7

(4) Seed germination medium

MS macrosalts
MS microsalts
FeEDTA (10^{-4} M)
Sucrose (1 wt%)
Agar (0.8 wt%)
Kanamycin. SO_4 (50 mg/liter)
pH 5.5

(5) Calcium washing solution

$CaCl_2 \cdot 2H_2O$ (0.16 M)
MES buffer (0.5 wt%)
pH 5.6

(6) Luria broth

Trypton 10 g/l
Yeast extract 5 g/liter
NaCl 10g/liter pH 7 with 1 m Tris-HCl
Gentamycin 50 mg/liter
Rifampicin 20 mg/liter

(7) Miller's macrosalts

KNO_3 1,000mg/liter
NH_4NO_3 1,000 mg/liter
$Ca(NO_3)_2$ $4H_2O$ 347 mg/liter
KH_2PO_4 300 mg/liter
KCl 65 mg/liter
$MgSO_4 \cdot 7$ H_2O 35 mg/liter

(8) MS microsalts: /7

$MnSO_4 \cdot 4$ H_2O 22.3 mg/liter
$ZuSO_4 \cdot 7$ H_2O 8.6 mg/liter
H_3BO_3 6.2 mg/liter
KI 0.83 mg/liter
$Na_2MoO_4 \cdot 2$ H_2O 0.25 mg/liter
$CuSO_4 \cdot 5$ H_2O 0.025 mg/liter
$CoCl_2 \cdot 6$ H_2O 0.025 mg/liter

Claims:

1. A method for gene transfer in plants, characterized in that

a) immature pollen grains are isolated from stamens in nutrient solution and the surrounding tissue is removed,

b) the isolated immature pollen grains are cultured in a nutrient solution,

c) foreign genetic material is transferred into the pollen grains during the in vitro culturing and maturation,

d) the transformed pollen grains are brought to complete maturation in vitro,

e) receiver plants are pollinated with the transformed pollen grains and seeds are obtained from said plants.

2. A method as recited in Claim 1, characterized in that the transfer of foreign genetic materials occurs in the stage of uninucleate microspores.

3. A method as recited in Claim 1, characterized in that the transfer of foreign genetic materials occurs during the first pollen mitosis.

4. A method as recited in Claim 1, characterized in that the transfer occurs in the early binucleate stage, as long as the generative cell is still attached to the pollen wall.

5. A method as recited in Claim 1, characterized in that the transfer of foreign genetic materials occurs shortly before or during the second pollen mitosis.

6. A method as recited in Claim 2, characterized in that the gene transfer occurs in the stage of uninucleate microspores in a medium enriched with sugar and other nutrient substances.

7. A method as recited in one of the Claims 1 through 6, characterized in that the transfer of foreign genetic materials occurs by co-culturing with *Agrobacterium tumefaciens*.